Determination of Fatty Acids in Small Amounts of Plasma and in Lipid Components of Tissues by Ultraviolet Spectroscopy

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METHODS for determining fatty acid composition of fats and fatty materials, based on ultraviolet absorption spectroscopy of the alkali-isomerized fatty acids and iodine values, have been employed extensively for the past decade. The application of these methods for fatty acid analysis of small amounts of plasma or other tissues, however, has received less attention. In this discussion an attempt is made to show how these methods can be successfully applied to determine polyunsaturated fatty acids in one milliliter or less of plasma, and in small fractions obtained in separation of tissue lipid components.

(A) DETERMINATION OF POLYUNSATURATED ACIDS IN PLASMA

Solvents: The type and purity of solvents for extraction of lipids from plasma or other tissues are extremely important. In our studies, it was found necessary to carefully redistill each solvent through a 30-inch Vigreux column to insure that it was free of nonvolatile residues and low in spectral density. The following absorbances of a number of solvents were found satisfactory.

Absorbances (1 cm cell/distilled water)

	220 mμ	233 mμ	268 mμ
Methanol	0.210	0.070	0.00
Methylal	0.560	0.260	0.00
"Iso"-octane*	0.333	0.037	0.00
Ethanol	0.175	0.062	0.00
Ethyl ether	0.720	0.252	0.015

* 2,2,4-trimethyl pentane.

For 0.5 to 2 ml of plasma: If only small amounts of plasma are available, the fatty acid analysis is limited to determination of polyunsaturated acids, owing to the small amount of lipid fatty acids represented. In these cases the alkali-isomerization and subsequent spectral analysis are conducted on the extracted lipid without weighing the lipid and without prior saponification and removal of unsaponifiable matter, as follows:

The measured or weighed plasma (0.5 to 2.0 ml, or 0.5 to 2 g) was added dropwise to 15 ml of Delsal[1] solvent (methylal-methanol, 4:1) in a 50 ml centrifuge tube with mechanical stirring. After stirring for 30 min, the solids were separated by centrifugation, and the clear solution was transferred to another 50 ml centrifuge tube and taken to dryness in a water bath at 55° C in a stream of nitrogen. The solids from the first extraction were extracted again with 10 ml of the solvent, centrifuged as before, and the second extract added to the lipid residue from the first extraction and taken to dryness. The total lipid residues were then extracted with two 10 ml portions of isooctane, the extracts clarified by centrifugation and transferred to a 25 ml volumetric flask. The solution was diluted to 25 ml and thoroughly mixed. Absorbances (before isomerization) were then determined with isooctane as blank at 233, 268, 315, 346, and 374 mμ and absorp-

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tivities were calculated at these wavelengths, the concentration being expressed in milliliters or grams of plasma represented by 1,000 ml of the lipid solution.

The isooctane solution of lipid was transferred quantitatively from the absorption cell and the volumetric flask to a 50 ml round-bottom flask and evaporated under nitrogen on a water bath at 60° C to about 3–5 ml volume. This concentrate was then transferred quantitatively with a dropping pipette to an isomerization tube,[2] which was clamped in a water bath held at 60° C, and the solvent was removed under nitrogen. (At least three 2 ml washes were used to transfer the lipid, at no time allowing the solution in the isomerization tube to exceed about 5 ml). When the lipid residue appeared dry, 2 g* (+0.02 g) of 21 per cent KOH-glycol reagent[2] was added to the isomerization tube containing the residue and to another tube as a blank. The tubes were flushed with nitrogen prior to addition of reagent, and, immediately after, were connected to the nitrogen flow and placed in isomerization bath at 180° C for 1 minute and shaken for 5 seconds in the bath with a back and forth motion. The shaking operation was repeated twice after 30-sec intervals. The total heating time in the bath was 16 minutes, after which the tubes were removed with nitrogen flowing and cooled in a beaker of cold water. The contents of the tubes were transferred quantitatively to 25 ml volumetric flasks with methanol, diluted to the mark, and thoroughly mixed. The absorbances were measured in the analytic spectral regions in the usual manner,[2,3] and the absorptivities calculated as before, again referring the concentration to the milliliters or grams of plasma represented by 1,000 ml of the lipid solution.

The initial absorptivity (before isomerization) at each analytical wave length was subtracted from that obtained after isomerization to give corrected absorptivities (a'_c). The latter, multiplied by 1,000 to express results in terms of mg per 100 ml or per 100 g, were substituted in the following formulae for (a) in the calculation of polyunsaturated acids.

Plasma, mg/100 ml

Hexaenoic acid $= 3.41a_{374}$

Pentaenoic acid $= 1.449a_{346} - 1.353a_{374}$

Tetraenoic acid $= 1.650a_{315} - 1.667a_{346} - 0.079a_{374}$

Trienoic acid $= 1.105a_{268} - 0.879a_{315} + 0.190a_{346} - 1.251a_{374}$

Dienoic acid $= 1.092a_{233} - 0.573a_{268} - 0.259a_{315} - 0.033a_{346} - 0.260a_{374}$

Comparisons were made of analysis of polyunsaturated acids of plasma obtained by the above procedure ("micro") with those obtained from a large quantity ("macro") of the same plasma (after saponification of extracted lipid and removal of unsaponifiables). In the latter case the fatty acids were weighed and the isomerization conducted on weighed portions of fatty acids. A summary of the comparison is shown by the following data:

Acid	"Micro" *mg/100 ml*	"Macro" *mg/100 ml*
Dienoic	43.2	40.1
Trienoic	3.9	3.6
Tetraenoic	11.5	10.0
Pentaenoic	3.9	3.0
Hexaenoic	Trace	1.4

If larger amounts of plasma (>2 ml) are available, the lipids may be extracted in similar manner by using proportionately greater amounts of solvent. The extracted lipid can be saponified, and the fatty acids recovered quantitatively and weighed. The weighed fatty acids may then be made up to a convenient known volume in isooctane, and aliquots taken for iodine numbers and spectrophotometric analysis, thus permitting calculations of oleic and saturated acids[5] as well as of polyunsaturated acids.

(B) DETERMINATION OF FATTY ACIDS IN LIPID COMPONENTS OF TISSUES

Silicic acid chromatographic adsorption technics for separating tissue lipids into their principal components, i.e., cholesterol esters, glycerides, free sterols, and phospholipids, have been

* The isomerization procedure and apparatus are the same as described previously,[2] except for the amount of reagent and total time of isomerization. Repeated analysis on a wide selection of different fats and oils by the modification showed good agreement with those obtained by the method as published.[2] The constants and equations are also the same except those for hexaenoic acids which are based on values published by Hammond and Lundberg.[4]

in use for over six years. However, very little has been published concerning the fatty acid composition of the separated fractions, possibly owing to the general lack of experience with the application of the alkali-isomerization spectrophotometric method to small samples. Although it appears that gas-liquid partition chromatography may eventually be the method of choice in determining the fatty acid composition, considerably more research is required to make it a practicable quantitative technic for the various polyunsaturated acids.

Fractionation of Lipids on Silicic Acid Columns: Following the work of Borgstrom,[6] a number of modifications of silicic acid chromatography have been employed for the separation of lipids.[7,8,9] Some of the modifications probably came about as a result of lack of uniformity in the activity of different grades and lots of silicic acid. In general, however, the order of separation and nature of eluting solvents used were similar, certain changes being required to effect the separation desired with the particular grade of silicic acid on hand. The following procedure is a modification found satisfactory in our laboratory. The apparatus was similar in general design to that described previously[10] for fractionation of methyl esters, except that only glass joints were used and the dimensions of the glass column were different. It was equipped for operation under constant pressure of nitrogen which, however, could be varied from 0 to several inches of mercury positive pressure. The glass column (21 mm x 300 mm) was packed by introducing a slurry of 22 g of silicic acid* and filter aid† (80:20) in 100 ml of redistilled petroleum ether (B.P. 35–60°). The slurry had been heated to boiling in a beaker with stirring for five minutes. During the addition of the slurry, slow stirring with a long thin stainless steel rod inserted in the column aided in uniformly packing the adsorbent as it settled by gravity. The adsorbent when settled in the column was about 190 mm in height. A receiver similar to that employed in collection of fractions under vacuum in distillations was used to collect measured volumes of eluate under nitrogen.

About 100 mg of weighed lipids in 10 ml of petroleum ether was added to the top of the column with an additional rinse of 10 ml of solvent. The first eluting solvent was then added to the separatory funnel, the space at top of column and the receiver flushed with nitrogen, and the flow rate adjusted to keep a constant level of liquid above the adsorbent. The drip rate through the column was adjusted to about 150 ml/hr by appropriate change of nitrogen pressure on the system. The order of elution and solvents employed were as follows:

(1) Hydrocarbons: 50 ml of petroleum ether + 1% ethyl ether.

(2) Sterol esters: 300 ml of petroleum ether + 1% ethyl ester.

(3) Glycerides: 300 ml of petroleum ether + 4% ethyl ester.

(4) Sterols + free acids: 350 ml of petroleum ether + 8% ethyl ester.

(5) Phospholipids: 250 ml of 3:1 ethyl ether-methanol followed by 250 ml of 1:1 ether-methanol.

In many instances, recovery of the material added to the column was substantially quantitative. Occasionally, some of the "phospholipid" components are not removed by this treatment and may require an additional treatment with chloroform-methanol 1:1. An alternate procedure in which the phospholipid fraction was first removed by precipitation with acetone and magnesium chloride solution (in ethanol) as described by Lipsky,[8] was found equally satisfactory. In this instance, the remainder of the lipid material in petroleum ether solution was put on the column, the order of separation of the other components being the same as before.

Treatment and Analysis of Eluted Fractions: The solvent was removed from the eluted fractions under reduced pressure of a water pump, and the lipid transferred quantitatively with small portions of petroleum ether to equilibrated and weighed (tared) 50 ml round bottom flasks. The solvent was then removed by

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* Mallinckrodt's Analytical Grade (100 mesh), "Suitable for Chromatographic Analysis by the Method of Ramsey and Patterson."

† Hyflo Super-cel filter aid. (The mention of commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others of similar nature not mentioned.)

evaporation under nitrogen, and finally under high vacuum and releasing to atmospheric pressure with oxygen-free nitrogen, until constant weight was obtained. The fractions were saponified with 0.5 N alcoholic KOH under nitrogen, the unsaponifiable material extracted with petroleum ether, and the fatty acids recovered by quantitative extraction after acidification with dilute sulfuric acid. The washed extract of fatty acids was taken to dryness and weighed as described for the eluted fractions; the fatty acids were then made up to standard volume with redistilled petroleum ether or isooctane. Aliquots were taken for determination of polyunsaturated acids by the alkali-isomerization spectrophotometric method as described for plasma lipids, except that in this case the weight of fatty acids is known and the concentration for calculating absorptivity is of course based on this weight.

The iodine values were determined on aliquots of the weighed fatty acids, representing 2 to 5 mg of the sample. The aliquots were added to 50 ml glass-stoppered flasks, the solvent removed under a stream of nitrogen, and 0.4 ml of chloroform added from a hypodermic syringe in such manner as to rinse the wall of the flask. Exactly 1.0 ml of 0.2 N Wijs solution was added with a micro-pipette, and the stoppered flasks were stored in a dark cabinet for 20 minutes. After this reaction time, the stoppers were loosened enough to permit washing by dropwise addition of 0.4 ml of 15 per cent KI solutiion followed by about 4 ml of distilled water. The liberated iodine was titrated with 0.01 N thiosulfate solution, 4 to 8 drops of 1 per cent starch solution being added as indicator.

The data in Tables I and II are presented primarily to serve as examples of the type of information obtainable by application of alkali-isomerization spectrophotometric analysis in conjunction with fractionating technics, such as chromatographic separation of lipids. Actually the spectrophotometric method should also prove to be useful adjunct in analysis of fractions obtained in gas chromatographic separations of methyl esters of fatty acids. Information of the type represented would appear to be valuable in connection with nutritional or biochemical investigations designed to show the effect of specific dietary fat constituents on lipid metabolism or in metabolic disorders.

TABLE I

Typical Separations of Component Lipids from Different Tissues

	A %	B %	C %	D %
Hydrocarbon	None	2.9	0.9	0.3
Sterol esters	28.0	40.3	19.9	1.2
Glycerides	19.4	14.1	9.9	13.4
Free sterols	38.9	8.3	3.0	3.2
Fatty acids	Trace	2.3	3.4	2.5
Phospholipids	13.3	32.2	62.4	78.2

A—Lipids from human atheromatous plaques.

B—Lipids from human plasma (healthy young men, post absorptive).

C—Lipids from rabbit plasma (adult males, post absorptive).

D—Lipids from rabbit liver (adult males, post absorptive).

TABLE II

Typical Analysis of Fatty Acids Obtained from Cholesterol Esters of Different Tissues

Acid	A %	B %	C %
Dienoic	23.6	47.4	49.5
Trienoic	1.4	0.7	1.7
Tetraenoic	7.0	8.0	3.0
Pentaenoic	1.3	1.0	0.4
Hexaenoic	1.0	0.7	None
Oleic	61.5	23.5	37.2
Saturated	4.2	18.7	8.2
Iodine values	134.0	142.7	139.3

A—Lipids from human atheromatous plaques.

B—Lipids from human plasma (healthy young men, post absorptive).

C—Lipids from rabbit plasma (adult males, post absorptive).

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